VESSEL FOR WITHDRAWING BLOOD

The present invention relates to a vessel for withdrawing blood, and the blood withdrawn should especially be used for stabilizing and analyzing nucleic acids.

When blood is taken, it is normally collected in vessels which already contain anticoagulants such as heparin, citrate or EDTA. The blood is thereby prevented from coagulating. The blood samples obtained thereby can be stored at suitable temperatures for a long time. This way of obtaining blood has, however, considerable drawbacks when nucleic acids such as (m)RNA or DNA are to be analyzed. For such purposes the nucleic acids contained in the sample should optimally be stabilized already at the moment of withdrawal, i.e. a degradation of the existing nucleic acids should be prevented, but also the new synthesis of mRNA.

This objective of a stable storage of the nucleic acids contained in the sample material, i.e. from the moment of withdrawal, has not been achieved yet in practice for the following reasons:
Cells contain nucleases, enzymes, which destroy nucleic acids as soon as they come into contact with the substrates thereof (RNA, DNA). The effect of cellular and extracellular nucleases is normally under physiological control as long as the cells are in their normal environment. The withdrawal of blood effects more or less strong changes in the nucleic acids contained in the cells. Nucleases are then released within the cells and/or by the lysis of cells to the outside. Moreover, nucleic acids are synthetized more or less strongly. In particular the long-term storage of blood leads to aging and destruction of the cells.

Another problem arising in the long-term storage of blood samples obtained according to standard withdrawal methods is the considerable change in the sample material. Such changes, e.g. strong lysis of cells, may have the effect that the standard methods for isolating nucleic acids no longer function in an adequately efficient way.

Apart from the problems regarding a stable storage of nucleic acids contained in the sample material, further difficulties arise in the conventional method for withdrawing blood. The conventional anticoagulants are often not separated efficiently enough during isolation of nucleic acids and interfere with in the subsequent analysis of nucleic acids, e.g. in the case of amplification by means of PCR (polymerase chain reaction). Heparin is e.g. a generally known inhibitor of PCR.

Finally, the question arises in the quantitative analysis of nucleic acids how the whole method ranging from sampling to the measurement of nucleic acids can be controlled under standardized conditions. Ideally, a quantitatively and qualitatively defined standard nucleic acid should already be added to the sample material during withdrawal and should be subjected to the whole process of sampling and determination. This can also not be accomplished with the conventional withdrawal systems.

A further drawback of conventional blood withdrawal is the risk of transferring infectious material because manual process steps have so far been needed for the isolation of nucleic acids. Contact with potentially infectious germs cannot be ruled out.

In the literature there is described a method in which the blood sample is mixed with guanidinium salt directly after withdrawal from a patient (EP 0 818 542 A1). In this method the guanidinium salt is present in powder form to thereby exploit the

increased stability of the guanidinium salt. This method, however, has serious drawbacks because the salt, for instance, must first dissolve in the added blood. This dissolution process depends, in particular, on the temperature and cannot be controlled because of the nontransparent sample material used. The use of a corresponding product for diagnostic medical purposes is thus very problematic.

Furthermore, nucleases are extremely active enzymes which can only be inhibited under extremely denaturing conditions. Denaturation depends on the concentration of the guanidinium salt in solution. An inhibiting concentration of guanidinium salt in solution does not exist in the cited method right from the beginning. Thus, there is an uncontrolled degradation of nucleic acids during the dissolution process. Moreover, in this method the addition of reducing agents is omitted, without which an efficient inhibition, in particular of RNases, is not ensured (see Example no. 5).

Moreover, the sample prepared in this way cannot directly be used for the further nucleic acid isolation on glass surfaces. Moreover, the use of guanidinium salt powder does not permit the addition of internal nucleic acid standards. Such standards are mandatory for process control and exact quantification.

The present invention has been based on the technical problem of providing a vessel for withdrawing blood which does not have the drawbacks of the prior art. In particular, it should be possible to subject the sample taken with the vessel directly to the standard methods for analyzing nucleic acids without the need for further sample preparation steps.

According to the invention this problem is solved by a vessel for withdrawing blood, the vessel containing an aqueous solution comprising the following components:

- a guanidinium salt;
- a buffer substance;
- a reducing agent; and/or
- a detergent.

The vessel of the invention has the following advantages: 1. Blood is already lysed at the moment of withdrawal in that the withdrawal vessel already contains a nucleic acid-stabilizing substance in solution. 2. The nucleic acid-stabilizing substance is composed such that the sample material, in particular the nucleic acids contained therein, are directly stabilized upon contact with the solution. 3. In contrast to all of the former standard withdrawal systems, such as EDTA or heparin-containing withdrawal vessels, the stabilized sample need no longer be handled as infectious material. 4. The nucleic acid-stabilizing substance is composed such that the sample material can directly be used in subsequent isolating methods. 5. The nucleic acid-stabilizing substance can be separated during subsequent isolation so efficiently that an inhibition of PCR is not observed. 6. The nucleic acid-stabilizing substance may have added thereto an internal standard. This permits the control of the whole method from the moment of sampling up to the detection of nucleic acids.

The withdrawal vessel mentioned under item 1 is a conventional blood withdrawing vessel (small tube) which has introduced thereinto a defined volume of a nucleic acid-stabilizing substance. The small tube is then preferably subjected to a defined vacuum which guarantees that only a specific volume of blood can flow thereinto during withdrawal. The small tube can be handled by conventional blood-taking methods. The solution contained in the tube contains the following reagents in a specially preferred embodiment: Guanidinium thiocyanate, Triton-X-100, dithiothreitol and a suitable buffer system, such as citrate, Tris or HEPES. In the described composition the solution is compatible with the vacuum tube. This solution can be stored in the vacuum tube without any problems and without any

impairment of the desired stabilizing function. The whole system presents no problems, in particular to blood donors, and is safe during sampling.

The solution containing the guanidinium salt, the buffer substance, the reducing agent and/or the detergent is stable in storage and converts the supplied and freshly taken blood into a material which is also stable in storage and can directly be subjected to the standard nucleic-acid analysis kits (e.g. those of Roche or Qiagen).

Guanidinium thiocyanate and/or guanidinium chloride are preferred as guanidinium salt.

Preferably, the guanidinium salt is present in a concentration of 2.0 to 8.0 M. Tris or citrate is preferred as the buffer substance, the exact pH being preferably adjusted with HCl. Further possible buffers are however HEPES, MOPS, citrate and phosphate buffer, such as PBS.

The buffer concentration is preferably between 10 and 300 mM, particularly preferably between 10 and 100 mM.

Triton-X-100 is preferred as the detergent. Further possible detergents are NP-40, Tween 20, polydocanol or other detergents.

The detergent concentration is preferably at 5 to 30% (w/v), particularly preferably at 10 to 20% (w/v).

DTT is preferred as the reducing agent, but ß-mercaptoethanol, TCEP (Tris(2-carboxyethyl)phosphine) or other reducing agents can also be used.

The preferred concentration of the reducing agent is at 0.1 to 10% (w/v), particularly preferred are 0.5 to 2% (w/v).

The pH of the solution is preferably at 3.0 to 9.0, particularly preferably at 4.0 to 7.5, particularly preferably at 5 to 6.

The pH of the solution is in particular chosen such that a pH ranging from 5.0 to 7.6 is set after addition of the sample material. Particularly preferred is a pH between 6.3 and 6.9 (see Example no. 8).

A particularly preferred solution preferably contains 4 M guanidinium thiocyanate, 45 mM Tris/HCl, 18%, preferably 15% (w/v) Triton-X-100, 0.8% (w/v) DTT and has a pH of 6.0.

In a further preferred embodiment the volume for receiving the blood sample has a negative pressure which can be adjusted such that a previously determined blood volume is sucked into the vessel after a blood vessel has been pierced. Correspondingly evacuated vessels are available on the market.

The vessel which contains the blood taken can then immediately be subjected to further analyses or, however, may be stored for a long period of time (up to several days) without any disadvantages for the quality of the sample.

In the method of the invention the freshly taken blood is directly contacted in the blood withdrawing vessel with the above-described solution so that all processes which might change the nucleic acid pattern of the sample are immediately stopped. Therefore, the data determined at a later time with respect to the detected nucleic acids very accurately represent the actual state at the time of blood withdrawal, i.e. both with respect to the quantities and the types of nucleic acids.

Preferably, the blood amount taken is 0.1 to 4 times the solution fed into the vessel. The solution is preferably 0.5 to 5.0 ml. Thus the final concentration of guanidinium salt after blood addition is at 1.0 to 5 M, preferably at 1 to 3 M, particularly preferred are 2-3 M (see Example 7).

The vessel according to the invention is preferably used for blood withdrawal when the blood sample is to be used for analyzing nucleic acids.

The use of the above-mentioned solution as a component of the described withdrawal system solely guarantees the immediate lysis of the cells and the simultaneous stabilization of the sample by immediate inactivation of the nucleases. Surprisingly, the blood sample obtained thereby can be stored even at room temperature or higher for several days. Moreover, the withdrawal system guarantees a contamination-free and non-infectious handling ranging from sampling via nucleic acid isolation to analysis. In the conventional methods of nucleic acid isolation, additional handling steps have so far been required (e.g. the transfer of the blood sample taken into the reagents for nucleic acid isolation, etc.), which entails an additional risk of infection.

The sample obtained with the blood withdrawing system is compatible with all of the conventional standard methods of nucleic acid isolation. Particular attention should here be paid to methods which are based on the binding of nucleic acids to glass surfaces, but also sequence-specific binding to complementary nucleic acid and solvent-based extraction methods.

Thus the invention as described consists of a blood withdrawing system which is conceived such that the following conditions are satisfied. 1. Controlled sampling and simultaneous stabilization of the nucleic acids (DNA, RNA) contained in the sample material. 2. Sampling in which the use of anticoagulants can be completely omitted. 3. The sample obtained by way of the above-described blood withdrawing

system can be used in a universal manner in all of the known systems for isolating nucleic acids. 4. The blood withdrawing system is stable in storage.

Additionally, it has surprisingly been found that the sample obtained by way of the described withdrawal system can be stored in the vessel for a long period of time without degradation of the nucleic acids (see Examples 2, 3, 7, 8).

The following examples will explain the invention:

Example 1:

The blood withdrawing system may be composed in a preferred embodiment as follows (see Fig. 1): A small tube is filled with a defined volume of the nucleic acid-stabilizing substance and is provided with a defined vacuum and sealed with a septum. The septum is constructed such that it is compatible with the standard sampling accessories (cannula, etc.). In the present example 2.2 ml reagent was supplied and the vacuum was adjusted such that exactly 2.2 ml blood could flow in during sampling. The nucleic acids contained in the inflowing blood flow were immediately converted into a stable form.

General preliminary remark regarding the following examples.

In all of the examples described hereinbelow, the nucleic acid-stabilizing substance (N-sS) had, unless indicated otherwise, the following composition: 45 mM Tris, 5 M guanidinium thiocyanate (GTC), 0.8% (w/v) dithiothreitol (DTT), 18% (w/v) Triton-X-100, pH 6.0.

In all of the examples described, the nucleic acid-stabilizing substance was, unless indicated otherwise, mixed with the sample in the ratio of 1 to 1 (1 volume N-sS

plus 1 volume sample material). A lower concentration of N-sS, e.g. 1 volume N-sS plus 5 volumes sample, might effect a degradation of RNA.

Blood was stabilized for all examples by directly feeding the blood upon withdrawal into the small tube mixed with N-sS.

Example 2:

Stability of nucleic acid after mixture of sample material and N-sS. Isolation of RNA and DNA from the sample lysate with silica-derivatized surfaces.

Material and method:

The sample material for the DNA and RNA isolation was directly used after withdrawal, after storage at 4°C for 6 days, and after storage at -20°C for 1 month.

The HighPure RNA Isolation Kit (Boehringer Mannheim, cat. no. 1828 665) was used for isolating RNA (Fig. 2). The instructions given in the package leaflet were modified as follows: A volume of 2.4 ml sample lysate was applied in 4 aliquots at 600 µl each to the column, so that a sample material of 2.4 ml lysate was applied on the whole. All of the other steps were carried out in accordance with the package leaflet. The RNA was finally eluted with 100 µl elution buffer.

For the isolation of DNA (Fig. 3) the QiaAmp Blood Kit (Qiagen cat. no. 29104) was used. The standard procedure described in the package leaflet was modified in various points: 400 µl sample volume was directly applied to the column; the binding reagent contained in the kit was not used. 25 µl proteinase-K batch solution was added and the sample was incubated at room temperature for 10 min. Subsequently, the column was put into a collection vessel and centrifuged as described in the package leaflet. All of the further steps were carried out in

accordance with the description in the package leaflet, except for the use of ethanol. The elution volume was 200 µl.

Example 3:

Isolation of mRNA from sample lysate using streptavidin-coated magnetic particles and biotin-labeled Oligo(dT) (Fig. 4):

Material and method:

20 ml sample lysate was fed into a vessel. The mRNA was isolated according to the following method: First of all 30 ml hybridization buffer (20 mM Tris-HCl, 300 mM NaCl, 6 nM biotin-labeled Oligo(dT), pH 7.4) was added to the lysate. 3 mg streptavidin magnetic particles (Boehringer Mannheim) were then added. The sample was mixed and incubated at room temperature for 5 min. The magnetic particles were separated with the help of a magnet; the supernatant was discarded. The particles were then resuspended in wash buffer 1 (10 mM Tris-HCl, 200 mM NaCl, 1% Triton-X-100, pH 7.5) and washed three times with wash buffer 2 (10 mM Tris-HCl, 200 mM NaCl, pH 7.5) (wash steps: resuspension, magnetic separation, removal of the supernatant). After the last wash step the supernatant was completely removed and the particles were resuspended in 20 µl distilled water. The sample was heated to 70°C for 5 min. The magnetic particles were separated, and the supernatant which contained the mRNA was analyzed by means of gel electrophoresis.

Example 4:

Isolation of DNA and RNA using a modified rule according to Chomczynski and Sacchi (Analytical Biochemistry 162, 156-159 (1987)) (example of a method based on solvent extraction) (Fig. 5):

Material and method:

2 ml sample volume was transferred from the blood withdrawing vessel into a small tube. 0.2 ml of a 2 M sodium acetate solution, pH 4, 2 ml phenol (water saturated) and 0.4 ml of a chloroform-isoamyl alcohol mixture (49:1) were then added, the sample being thoroughly mixed after addition of each solution. The complete solution was vigorously shaken for 10 seconds and incubated on ice for 15 minutes. The sample was centrifuged for 20 minutes at 4°C at 10000 g. After centrifugation the RNA was in the aqueous phase; the DNA and proteins in the intermediate and phenol phase. The aqueous phase was transferred into a new vessel and mixed with 1 ml isopropanol. For precipitating the RNA the sample was stored at -20°C for 1 hour. After renewed centrifugation at 4°C at 10000 g the RNA was pelleted. The pellet was resuspended in 0.3 ml buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, 0.1 M 2-mercaptoisopropanol), transferred into a new 1.5 ml Eppendorf vessel and mixed with 1 volume of isopropanol. After incubation at -20°C for 1 hour the solution was centrifuged in an Eppendorf centrifuge at 4°C for 10 minutes. The RNA pellet was received in 75% ethanol and concentrated by centrifugation (Speed vac) and dried. For further processing the sample was dissolved in 100 µl 10 mM Tris-HCl, pH 6.5.

Example 5

Importance of reducing reagents (such as DTT) in the stabilizing solution for the long-term stability of RNA

Material and method:

Stabilizing solution used: 4.0 M GTC; 13.5% Triton X100; 45 mM Tris/HCl; with or without 120 mM DTT. 700 µl serum was mixed with 700 µl stabilizing solution. After incubation for 2 min 20 µl MS2-RNA (0.8 µg/µl of Roche) was added. The samples were incubated at 40°C for 180 min and then processed in aliquots of 400 µl each with the High Pure total RNA Kit of Roche. The samples were applied in one step to the column without addition of the binding reagent of the kit and centrifuged in accordance with the instructions. The following wash steps and the elution of the RNA in 50 µl elution buffer were carried out in accordance with the instructions.

The analysis was carried out by means of agarose gel (see Fig. 6).

Result: Without the addition of reducing reagents to the stabilizing solution no long-term stabilization of RNA can be achieved.

Example 6

Stability of free MS2-RNA in serum. Kinetics of the RNA degradation by sample components.

Material and method:

250 µl serum was spiked with 10 µl MS2-RNA (0.8 µg/µl of Roche) and incubated at room temperature. Immediately after the addition of RNA, after 2 min to 50 min, the natural RNA degradation in serum was stopped by adding 250 µl stabilizing solution. All batches were analyzed twice. As a standard, a sample was only mixed with MS2-RNA after addition of the stabilizing solution to the serum and was processed in parallel.

All samples were processed in parallel with the High Pure viral RNA Kit of Roche. The samples were applied to the column in one step without addition of the binding reagent of the kit and centrifuged according to instructions. The following

wash steps and the elution of RNA in 50 µl elution buffer were carried out according to instructions.

20 µl of the eluate was separated by means of a 1.2% native agarose gel and analyzed (see Fig. 7).

Result: MS2-RNA is not stable in serum. Already 2 minutes after addition of RNA to the serum the RNA is completely degraded. By the addition of stabilizing solution to the serum in the ratio of 1:1, this process can be stopped immediately, and a stabilization of the RNA can be achieved at the time when the stabilizing solution is added (= blood withdrawal).

Example 7

Stability of MS2-RNA in serum/stabilization solution. Dependence on the GTC concentration

Material and method

Stabilization solutions used: 3-5 M GTC; 13.5% Triton X100; 50 mM DTT; 42 mM Tris/HCl

pH of the solutions: about 4.0

pH of the solutions after addition of serum: about 6.7.

2 ml serum was mixed with 2.5 ml of the respective stabilization solutions. After an incubation time of 2 to 5 min 90 µl MS2-RNA (0.8 µg/µl of Roche) was added and incubated at 40°C. 400 µl samples were taken at regular intervals and processed with the High Pure total RNA Kit of Roche according to Example 5. The samples were eluted in 50 µl and frozen at -20°C. For the analysis of the RNA integrity 20 µl of the eluate was applied to a 1.5% agarose gel (see Fig. 8).

For the PCR analysis of the samples 10 µl of the eluate was reversely transcribed by means of AMV-RT (Roche) and subsequently analyzed by means of PCR on the Lightcycler:

Batch for RT:	4.0 μl	AMV-RT buffer
(42°C for 1 h)	2.0 μl	dNTP's (final concentration 10 mM)
	0.5 μl	RNase inhibitor (Roche, 20 units)
	1.0 μl	Primer 2827 (final concentration 1 μM)
	1.9 μl	DMPC water
	0.6 μl	AMV-RT (Roche, 15 units).
	10 μl	template RNA
	Σ 20 μl	

The PCR was carried out on the Lightcycler at an annealing temperature of 61°C using SYBR-Green as detection system. Batch for PCR:

1.6 μl	MgCl₂ (batch solution 25 mM)
5.9 μl	DMPC water
0.25 μl	Primer 2827 (batch solution 20 mM)
0.25 μl	Primer 2335 (batch solution 20 mM)
1.0 μl	SYBR-Green-Mastermix (Roche)
1.0 μl	RT batch (1:50 diluted)
Σ 10 μl	

The amplificate of the PCR was completely applied to a 2% agarose gel (see Fig. 9).

Result:

RNA integrity at 40°C after 3 days.

The agarose gel in Fig. 8 shows 20 μl of the eluted MS2-RNA after incubation at 40°C for 3 days. After this period distinct differences in the RNA integrity can be made out in dependence upon the GTC content. Thus a salt content of less than

2 M in the serum/stabilization solution is of advantage to the integrity of the RNA.

Amplificability of the RNA at 40°C after 8 days.

Although a beginning degradation of the RNA was already detected at 40°C after 3 days, all of the RNA samples could be amplified after an incubation of 8 days at 40°C and clearly detected.

The amplificate of the PCR was fully applied to a 2% agarose gel (see Fig. 9).

Example 8

Stability of MS2-RNA in serum/stabilization solution: dependence on the pH of the sample mixed with stabilization solution.

Material and method:

Solution used: 4 M (5 M)   GTC

14.4%   Triton X 100

50 mM   DTT

45 mM   Tris/HCl

pH after serum addition between 6.7 and 8.0

2.5 ml stabilization solution was mixed with 2.0 ml serum. After addition of 90 µl MS2-RNA (0.8 µg/µl, Roche) the samples were incubated at room temperature. The RNA was processed at regular intervals from 500 µl sample with the Roche viral RNA Kit according to Example 6 and isolated in 50 µl elution buffer.

20 µl of the eluate was analyzed by means of agarose gel (see Fig. 10).

Result:

The pH of the serum/stabilization solution and thus the pH and the buffer range of the stabilization solution are decisive for the long-term stabilization of RNA. While

at a pH of 8.0 an intact RNA could no longer be detected already after 2 days, intact RNA is still detectable within a pH range between 6.6 and 7.0 after 13 days of incubation at room temperature.

Apart from the pH, however, an optimally adjusted GTC concentration is also of importance to the long-term stabilization of RNA (see also Example 7). The illustrated example demonstrates that a GTC final concentration in the stabilized sample of 2.2 M GTC is better than 2.78 for a long-term stabilization of RNA.

Legends

Fig. 1:

Sampling vessel with N-sS, defined vacuum, sealed with septum.

Fig. 2:

Gel analysis (1% agarose) of RNA which was stored in the sampling vessel for different periods of time. Column 1: Isolation directly after sampling (no storage), column 2: storage for one month at -20°C, column 3: storage for 6 days at 4°C. The amount of the applied RNA corresponded to a blood volume of 120 µl.

Fig. 3:

Gel analysis (1% agarose) of DNA which was stored in the sampling vessel for different periods of time. Column 1: isolation directly after sampling (no storage), column 2: storage for one month at -20°C, column 3: storage for 6 days at 4°C. The amount of the applied DNA corresponded to a blood volume of 10 µl.

Fig. 4:

Gel analysis (1% agarose) of mRNA which was isolated from 10 ml blood (column 2). Molecular weight marker (column 1). In addition to the mRNA, the rRNA bands are visible. The sharp contours of the bands demonstrate the integrity of the nucleic acids.

Fig. 5:

Gel analysis (1% agarose) of the RNA which was isolated from 120 µl blood.

Fig. 6:

Gel analysis of isolated MS2-RNA after incubation in serum/stabilization solution with/without DTT for 180 min at 40°C.

Column 1: positive control: MS2-RNA, column 2: DNA marker, column 3,4,5: MS2-RNA after incubation with DTT-containing stabilization solution, column 6,7,8: MS2-RNA after incubation with stabilization solution without DTT.

Fig. 7:

Gel analysis of isolated MS2-RNA after incubation in serum for 0-50 min

Column 10,17: MS2-RNA standard, column 9,16: DNA marker, column 7,8: incubation for 0 min, column 5,6: incubation for 2 min, column 3,4: incubation for 5 min, column 1,2: incubation for 10 min, column 11,12: incubation for 15 min, column 13,14: incubation for 30 min, column 15: incubation for 50 min

Fig. 8:

Gel analysis of MS2-RNA which was isolated after incubation in serum/stabilization solution for 3 days at 40°C. The GTC content of the stabilization solution after serum addition in which the relevant RNA sample was incubated is indicated in the corresponding column.

Column **1**: 2.70 M GTC, column **2**: 2.5 M GTC, column **3**: 2.36 M GTC, column **4**: 2.20 M GTC, column **5**: 2.08 M GTC, column **6**: 1.94 M GTC, column **7**: 1.80 M GTC, column **8**: 1.66 M GTC.

Fig. 9:

Gel analysis of the PCR amplificates of MS2-RNA which was isolated after 1 day and 8 days, respectively, of incubation at 40°C in serum/stabilization solution.

Column **1**: Amplificate of the RNA isolated after 1 day, column **2**: amplificate of the RNA isolated after 8 days, column **3**: DNA marker, column **4**: MS2-RNA positive control: 0.8 µg in 10 µl RT, 1:50 diluted, 1 µl amplified.

Fig. 10:

Gel analysis of isolated MS2-RNA after 6 days (column **2-12**) and 13 days (column **14-19**), respectively, of incubation at room temperature in serum/stabilization solution. The pH which was obtained after mixing of serum and stabilization solution is written behind the corresponding columns.

Column **1, 13, 20**: DNA marker, column **2**: pH 8.0, column **3**: pH 7.7, column **4**: pH 7.5, column **5**: pH 7.35, column **6**: pH 7.18, column **7,14**: pH 7.07, column **8,15**: pH 6.94, column **9,16**: pH 6.8, column **10,17**: pH 6.72, column **11,18**: pH 6.68 and column **12,19**: pH 6.7. The stabilization solution of RNA in column **12, 19** had the same pH as that of the RNA in column **11**, but contained 5 m GTC instead of 4 M.